P34^{cdc2} kinase is associated with cortical microtubules from higher plant protoplasts

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Abstract The cell cycle regulatory enzyme p34^{cdc2} kinase is known to be localized to the preprophase band, the spindle and the phragmoplast, but not to interphase cortical microtubules. This was investigated further by mechanically cleaving substrate-attached protoplasts to leave plasma membrane disks bearing microtubules freed of nuclear and cytosolic signal. Antibodies to PSTAIRE and to specific C-terminal peptides of cdc2a, were used in immunofluorescence, protein blotting and immunogold electron microscopy to demonstrate that antigen is located on the cortical microtubules of carrot, tobacco BY-2 and *Arabidopsis* cells.

Key words: PSTAIRE; cdc2; Microtubule; Plant cell; Cytoskeleton

1. Introduction

The passage of a cell through the division cycle is regulated by cyclin-dependent protein kinases (CDKs) activated at key transition points by association with various cyclins [1]. The prototypical CDK, cdc2/CDC28, was originally described in yeast and functional homologs were subsequently found to occur throughout phylogeny. In plants, various CDK-like proteins have been cloned in a range of species, the best-characterized belonging to the A-type class. Members of this class share the PSTAIRE motif in the cyclin-binding domain, and they most resemble yeast cdc2/CDC28 which 'starts' the division cycle (reviewed in [2]).

Activated CDKs alter the structure of cells by phosphorylating skeletal proteins. In animal cells, cdc2-dependent phosphorylation of lamins initiates the breakdown of the nuclear envelope as a prelude to mitosis [3]. It also converts interphase microtubules [4] to the shorter more dynamic MTs characteristic of mitosis [5] by affecting the microtubule-stabilizing and -nucleating properties of microtubule-associated proteins (MAPs) [6,7]. In animal cells,there is therefore firm evidence that links CDKs to the MT cytoskeleton.

In plants, cyclin-dependent processes also drive the plant MT cycle and several studies show that CDKs are associated with MTs. Mineyuki et al. [8] showed that anti-PSTAIRE labelled the preprophase band (PPB) of onion root tip cells. Colasanti et al. [9] then confirmed the PPB localization using an antibody raised to a C-terminal peptide from maize cdc2. Next, Mineyuki et al [10] demonstrated that variations in the stainability of the PPB produced by the two methods could be attributed to the conditions of fixation. Stals et al. [11] used a polyclonal antiserum to immunolocalize plant cdc2 kinase in alfalfa root tip cells. They confirmed the PPB staining and went on to show that the metaphase spindle, the late anaphase spindle and phragmoplast could also be stained by both anti-PSTAIRE and anti-cdc2 peptide antibodies. The activating components of the CDK complex, the cyclins, also co-localize with MTs [12]. Not only was cyclin IB found on the PPB but cyclin II was found to be associated with all MT arrays, including the interphase array; this was suggested to be correlated with the continuing active turnover of cortical microtubules even in non-dividing cells [13]. This agrees with the finding that cyclin IB occurs on cytoplasmic microtubules in animal cells [7,14,15] and invites the question of whether CDKs, which partner the cyclins, might similarly be located on plant cytoplasmic microtubules.

By exploiting substrate-attached plasma membrane disks ('footprints' [16]) which bear microtubules freed of most other cellular material,we show that p34^{cdc2} kinase is located on cortical microtubules. Using this method, cortical microtubules could be stained with antibodies to PSTAIRE as well as with antibodies to the C-terminal region of cdc2a. Immunogold electron microscopy of pre-fixed footprints confirmed that the PSTAIRE antigen specifically decorates microtubules rather than any other associated organelle. We discuss the potential roles that p34^{cdc2} kinase might have on the highly dynamic cortical microtubules.

2. Materials and methods

2.1. Cells

Tobacco BY-2 cells, an *Arabidopsis* cell suspension, and a carrot suspension were converted to protoplasts with 2% (w/v) cellulase R10 (Onozuka), 0.05% (w/v) Pectolyase Y23 (Yakult) in 0.4 M sorbitol in PME buffer (50 mM PIPES, pH 6.9, 5 mM $MgSO_4$, 5 mM EGTA) for 3 h.

2.2. Footprints

To make footprints, protoplasts were treated with 20 μM taxol and settled for 20 min onto poly-L-lysine-coated multiwell slides. They were held vertically in 80 ml PME plus 7 μg/ml PMSF and 1 μg/ml leupeptin, then burst by touching against another slide, fixed in 4% (w/v) formaldehyde in PME and washed in buffer. The following anti-

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Abbreviations: PPB, preprophase band; CDK, cyclin-dependent kinase; MAP, microtubule-associated protein

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bodies were then added at 4°C overnight in PME plus 3% (w/v) bovine serum albumin (BSA) before being washed and incubated with respective Cy3- or Alexa 568-conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature: anti-α tubulin (YOL1/34) (anti-β tubulin (Sigma) was used for blotting), anti-PSTAIRE (Calbiochem (rabbit) and Sigma (mouse)) and anti-cdc2 peptide antibodies (kind gift of Dr. J. Murray, University of Cambridge [17]) against the ARAALEHEYFKDLGGMP sequence of *Arabidopsis* cdc2a (termed M169); and M171 against the ARNALEHEYFKDIGYVP sequence of tobacco cdc2a.

For immunoblotting, footprint preparations on glass slides were dissolved in ×4 SDS sample buffer, separated by SDS–PAGE and transferred to nitrocellulose.

To investigate the distribution of antigen at the electron microscopic level, BY-2 protoplasts (which produced the best footprint preparations) were settled onto plastic/carbon-coated grids, converted to footprints by touching against another grid under excess buffer. To control against the redistribution of enzyme upon bursting, adherent protoplasts were fixed in 4% (w/v) formaldehyde before bursting and no detergent was used at any stage. After cleaving, footprints were washed in 5% (w/v) BSA in PME buffer before receiving the primary antibody and the colloidal gold-conjugated secondary antibody. They were then post-fixed in glutaraldehyde and tannic acid, stained with osmium tetroxide and uranyl acetate, and finally dehydrated through an ethanol series before being critically point dried.

3. Results

3.1. Immunolabelling of cortical microtubules on footprints

Figure 1 shows that two commercial anti-PSTAIRE antibodies, raised in different animal species, produced a microtubule-like staining pattern for carrot, tobacco BY-2 and *Arabidopsis* preparations. The two antibodies raised against non-PSTAIRE, specific C-terminal epitopes in BY-2 and *Arabidopsis* cdc2a also produced linear staining patterns which are therefore produced, in various combinations, by four different antibodies, using different secondary antibodies, on three different cell types. By themselves, secondary antibodies produced no such patterns. To confirm that these staining patterns were dependent upon the presence of microtubules, protoplasts were treated for 1 h with microtubule-specific drugs before making footprint preparations. Fig. 2 shows that after taxol treatment, anti-PSTAIRE antibodies produced the linear cortical staining pattern, but when protoplasts were pre-treated with oryzalin to depolymerize microtubules, the staining was reduced accordingly.

3.2. Immunoblotting a ca. 33 kDa antigen in protein extracts of footprints

To demonstrate that the antibodies were recognizing a ca. 34 kDa protein, footprint preparations were dissolved in ×4 SDS sample buffer and examined by immunoblotting (Fig. 3). Anti-PSTAIRE blotted a 33 kDa band in these samples (PSTAIRE, lanes A and B). The M169 antibody against a C-terminal peptide of *Arabidopsis* cdc2a also recognized a 33 kDa band (M169, lane A) from *Arabidopsis* footprints, while the M171 antibody raised against a peptide in the C-terminus of tobacco cdc2a showed a positive cross-reaction using a BY-2 preparation (M171, lane B). None of the four different antibodies used recognized tubulin. In the upper two

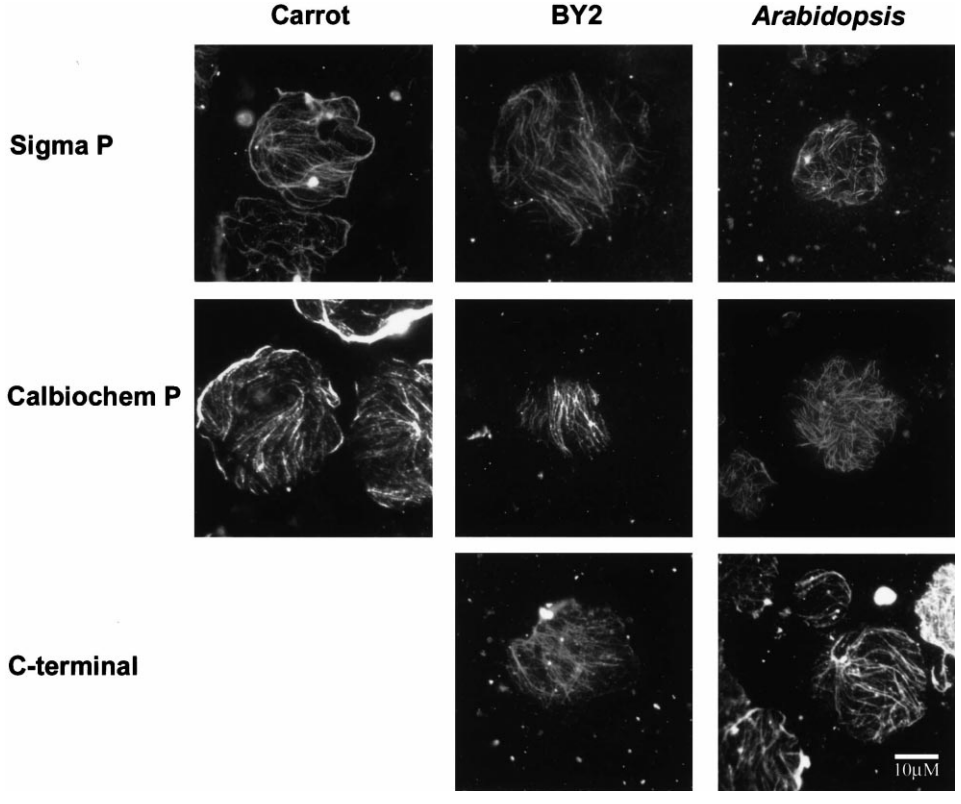

Fig. 1. Immunostaining of cortical microtubules by anti-cdc2 antibodies. Antibodies to PSTAIRE, from Calbiochem and Sigma, fluorescently labelled MTs in all three cell types. Antibodies to the C-terminal cdc2a peptides of tobacco (M171) and *Arabidopsis* (M169) also stain cortical MTs in these species. Scale bar = 10 μm.

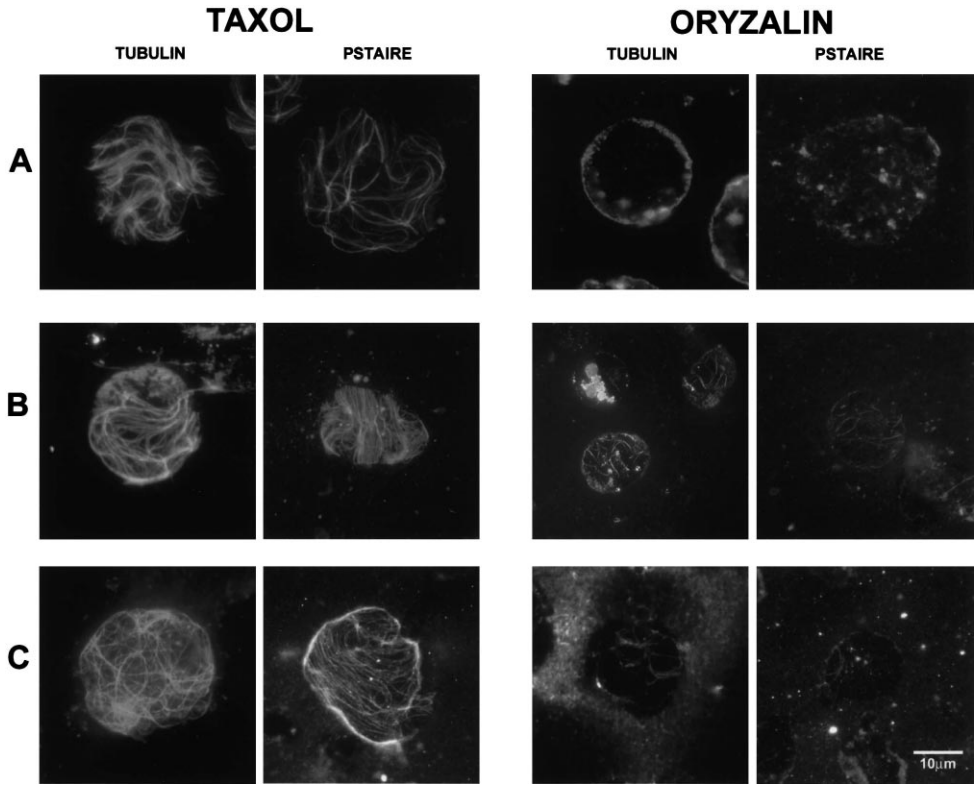

Fig. 2. Effects of anti-microtubule drugs. The cortical MTs of taxol-treated *Arabidopsis* (A), BY-2 (B) and carrot (C) were stained with anti-PSTAIRE or anti-tubulin. In contrast, the anti-microtubule herbicide oryzalin removed MTs and PSTAIRE signal also.

lanes, anti-β tubulin controls for *Arabidopsis* and BY-2 samples show the presence of tubulin in the samples.

3.3. Immunoelectron microscopy of footprints

BY-2 protoplasts produced the best and most consistent footprints and were therefore used for higher resolution studies at the electron microscopic level. These were fixed first in formaldehyde before cleaving. The immunogold labelling in Fig. 4 shows that PSTAIRE antigen was localized to microtubules with a high degree of specificity: they were decorated along their length as well as on material bridging adjacent microtubules (Fig. 4, see inset). No gold particles were observed on the plasma membrane, the tubulo-reticular endoplasmic reticulum, coated pits or coated vesicles.

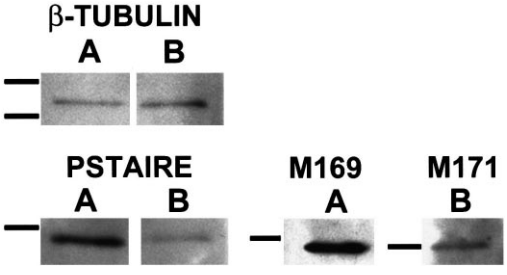

Fig. 3. Immunoblotting footprint extracts with anti-cdc2 antibodies. The presence of microtubule protein in SDS-dissolved footprint extracts was confirmed by blotting with anti-β tubulin (markers 62 and 48 kDa). Anti-PSTAIRE recognized a 33 kDa band in *Arabidopsis* (A) and BY-2 (B) extracts. The C-terminal peptide antibody to *Arabidopsis* cdc2a (M169) recognized a 33 kDa band in *Arabidopsis* (A); similarly, the C-terminal peptide antibody against BY-2 cdc2a (M171) recognized a band in the tobacco BY-2 extract (B).

4. Discussion

This study has shown that anti-PSTAIRE and anti-cdc2 peptide antibodies immunolabel cortical MTs on footprints made from carrot, tobacco and *Arabidopsis* protoplasts. Previous studies have shown that antibodies to CDKs stain the PPB [8–10] as well as the spindle and phragmoplast [11,12] Although these papers have noted signal in the interphase cytoplasm, this is the first to show association with cortical microtubules. One reason for this could be that unlike the PPB, kinetochore fibers and the phragmoplast – in which microtubules are bundled –, the cortical microtubules are evenly distributed over a large area, making it difficult to discern signal against the diffuse cytosolic background. Removal of the cytosolic pool during footprint formation should favor visualization of a subpopulation of $p34^{cdc2}$ on the cortical microtubules. In unpublished studies on carrot cells (Bush, Doonan and Lloyd), we have found that whilst most of $p34^{cdc2}$ is extractable with detergent, some is retained on the detergent-insoluble cytoskeleton, supporting the idea of different pools. In the present study, care was taken to control against possible redistribution of antigens during processing. No detergent was used that might allow nuclear PSTAIRE antigen to pass into the cytoplasm or for other membrane-bound pools to be redistributed. Instead, protoplasts were mechanically burst under buffer. As a further control, substrate-attached protoplasts were pre-fixed before cleaving in the immunogold studies: the highly specific gold labelling of MTs observed in the electron microscope, which did not extend to any other cortical organelle, demonstrated that bursting did not cause promiscuous scattering of antigen.

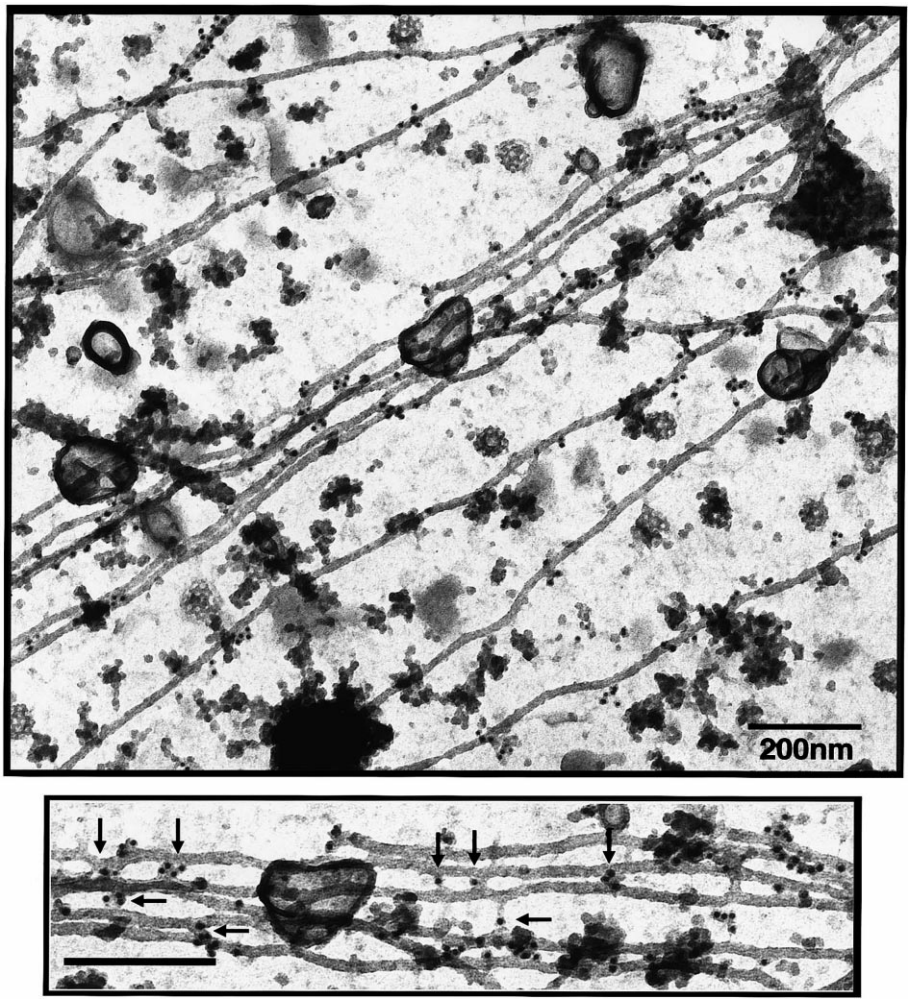

Fig. 4. Immunogold labelling of BY-2 cortical MTs with anti-PSTAIRE. Antibody–gold particles specifically decorate microtubules, but not coated pits and vesicles, endoplasmic reticulum nor plasma membrane. Arrows in the lower inset point to labelling of material bridging parallel microtubules.

Another possibility is that the interaction of cytoplasmic $p34^{cdc2}$ with microtubules is stimulated by protoplast formation. In animal cells, cytosolic mitogen-activated protein kinase becomes associated with cytoplasmic microtubules when activated by signals [18,19]. Such shuttling between cytosolic and cytoskeletal pools was recently reported for alfalfa root cells in which a stress-inducible MAP kinase was shown to become re-located to the PPB and phragmoplast of some cells [20], possibly in response to hyperosmotic or mechanical stress. It is conceivable that the association of $p34^{cdc2}$ with cortical MTs seen in the present study might also be a response to the stress of protoplast formation. However, another explanation for the difference between this and previous studies is that we have used suspension cells in contrast to the tissue cells used elsewhere. Suspension cells maintained in the division cycle by regular subculturing might be expected to have different patterns of expression of cyclins and CDKs. Compared to primary and secondary root meristems, non-dividing tissue cells have a persistently low amount of $p34^{cdc2}$ protein, speculated to be available for possible further dedifferentiation [2,13]. Indeed, this is increased in tissue cells when they respond to wounding by dividing. Similarly, non-dividing legume root cells have been hypothesized to retain some inactive cdc2/cyclin in order to respond rapidly to nodulation factors [21]. However, in suspension cells frequently subcultured into medium containing plant growth hormones, the amount of cdc2 protein is constitutively high irrespective of the actual cell cycle phase [22–24] and is only increased a little when BY-2 cells re-enter the cell cycle at the G_1/S transition [25].

It has been suggested that cyclins persist in non-dividing plant cells in order to participate in processes such as microtubule turnover [2,13]. If so, then it is reasonable to suppose that the enzymatic half of the cdc2/cyclin complex could have some association with interphase microtubules and be part of the general mechanism that coordinates the transitions of the microtubule cycle with the DNA replication and separation cycle. There is ample evidence from animal studies that cdc2 kinase affects microtubule dynamics by being associated with, and phosphorylating, microtubule-associated proteins (e.g. [4–7]). Despite forming more-or-less parallel arrays in which cortical microtubules are held in register by cross-bridging proteins like MAP65 [26], the interphase array can, by de- and re-polymerization, reorient itself through 90° in as little as 18 min [27]. Even those microtubules that are not reorienting in response to factors such as hormones have been shown

to exchange their tubulin subunits with half-times of as little as 60 s [28,27]. Hence, despite the high degree of order, the microtubule array is highly dynamic. It is possible, therefore, that some of the cdc2 kinase, constitutively present throughout the cell cycle, and known to be capable of binding to microtubules in all other three arrays, becomes located to the cortical microtubules of suspension cells, either as a reflection of their constant mitogenic stimulation, in readiness for the G_1/S transition, and/or as part of the mechanism that keeps cortical MTs dynamic in readiness for reorientation.

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